Pressor Effect of Blocking Atrial Natriuretic Peptide in Nucleus Tractus Solitarii

Ren-Hui Yang, Hongkui Jin, J. Michael Wyss, Yiu-Fai Chen, and Suzanne Oparil

Previous studies have shown that microinjection of atrial natriuretic peptide into the caudal nucleus tractus solitarii produces significant increases in local neuronal firing rate associated with reductions in arterial pressure in anesthetized Wistar rats. Single units excited by microinjection of atrial natriuretic peptide into the caudal nucleus tractus solitarii were also excited by activation of arterial baroreceptors and inhibited by baroreceptor unloading. To test the hypothesis that endogenous atrial natriuretic peptide in caudal nucleus tractus solitarii is involved in the tonic control of blood pressure in the rat, we administered a blocking monoclonal antibody to atrial natriuretic peptide in a volume of 50 nl artificial cerebrospinal fluid via microinjection into the caudal nucleus tractus solitarii of spontaneously hypertensive and Wistar-Kyoto rats and observed the effects on mean arterial pressure and heart rate. Control injections of monoclonal antibody were administered into the rostral nucleus tractus solitarii, hypoglossal nucleus, spinal trigeminal nucleus, and cuneate nucleus of spontaneously hypertensive rats. Microinjection of monoclonal antibody into the caudal nucleus tractus solitarii caused significant increases in mean arterial pressure in spontaneously hypertensive rats but not in Wistar-Kyoto rats. There was no concomitant change in heart rate. Control injections of purified mouse immunoglobulin into the caudal nucleus tractus solitarii and of monoclonal antibody into the control neuronal groups listed above had no effect on mean arterial pressure. These results suggest that endogenous atrial natriuretic peptide in the caudal nucleus tractus solitarii mediates tonic control of blood pressure in spontaneously hypertensive rats but not in normotensive Wistar-Kyoto rats. (Hypertension 1992;19:198–205)

Atrial natriuretic peptide (ANP), originally described in mammalian atria, is also found in brain. Immunoreactive ANP has been detected in various brain regions by radioimmunoassay of extracted tissue and by immunocytochemistry.1–5 ANP messenger RNA (mRNA) and ANP receptors have also been described, indicating that an intact ANP synthetic, processing, and effector system is present in rat brain.6–10

The nucleus tractus solitarii (NTS) plays an important role in the central regulation of blood pressure by influencing sympathetic nervous system activity and vasopressin release.11 The caudal NTS is the primary site of termination for carotid and aortic baroreceptor afferents. Microinjection of exogenous ANP into the caudal NTS has been shown to produce significant decreases in blood pressure and increases in firing rate of NTS neurons in urethane-anesthetized normotensive Wistar rats, suggesting that ANP-induced activation of NTS neurons may mediate depressor responses.12,13 These findings suggest the possibility that tonic activation of NTS neurons by endogenous ANP may be involved in blood pressure regulation.

The current study tested the hypothesis that blockade of endogenous ANP in caudal NTS by local microinjection of a monoclonal antibody to ANP (MAB KY-ANP-II) increases blood pressure by blocking tonic activation of NTS neurons by endogenous ANP. We studied spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats maintained on basal NaCl diets. Control microinjections of MAB KY-ANP-II were delivered into hypoglossal nucleus, spinal trigeminal nucleus, cuneate nucleus, and rostral NTS. These control regions were selected because of their proximity to the caudal NTS, their involvement in cardiovascular control, or both. Microinjections of immunoglobulin G (IgG) purified from mouse ascites fluid into caudal NTS served as vehicle controls. Our results demonstrated that mi-
Microinjection of a blocking monoclonal antibody to ANP into the caudal NTS but not into the rostral NTS or any other control region examined produced significant increases in blood pressure in SHR but not in WKY rats. Microinjection of IgG into the caudal NTS did not alter blood pressure in SHR. These results suggest that endogenous ANP in the caudal NTS mediates tonic control of blood pressure in SHR but not in normotensive WKY rats.

Methods

SHR and normotensive WKY control rats were obtained from Taconic Farms (IBU-3 colony, Germantown, N.Y.) at 7 weeks of age. All rats were maintained four per cage at constant humidity (65±5%), temperature (24±1°C), and light cycle (6 AM to 6 PM). Rats were provided a standard rat diet (5001, Ralston-Purina, Richmond, Ind.) and free access to food and water. For 1 week before acute study, rats were aclimated to these diet and housing conditions. All procedures followed in these experiments were in accordance with institutional guidelines and were approved by the University of Alabama at Birmingham Animal Use Review Committee.

Two days before the acute experiment, each rat was anesthetized with sodium pentobarbital (50 mg/kg i.p.), and a cannula (polyethylene PC-10 fused with PE-50) was implanted into the abdominal aorta in anesthetized SHR and WKY rats. Microinjection experiments were carried out in conscious, freely moving rats. Transient lability of heart rate and blood pressure during the initial stabilization period appeared to be the same in WKY and SHR and to be a result of handling rather than a consequence of cannula placement per se.

In a separate series of experiments, muscimol (20 µg), a direct acting γ-aminobutyric acid agonist, was microinjected in a volume of 50 nl dissolved in ACSF into the caudal NTS of WKY rats as a positive control, since microinjection of MAb KY-ANP-II into the caudal NTS did not affect the mean arterial pressure of WKY rats. Previous studies by other investigators have shown that microinjection of muscimol in this dose into NTS caused pressor responses in anesthetized SHR and WKY rats. At the conclusion of each experiment, 1% methylene blue solution (50 nl) was injected through the cannula. The rat was anesthetized with sodium pentobarbital (60 mg/kg i.p.), decapitated, and the cannula was removed from the brain. The brain was removed from the skull and sectioned at 30 µm on a freezing microtome (Slee Medical Equipment Ltd., London, UK). Sections were

Figure 1. Left panel: Diagrammatic representation of a coronal section through the medulla at a level 4.8 mm caudal to the interaural line. Increases in mean arterial pressure were observed only in response to microinjection of monoclonal antibody to atrial natriuretic peptide (MAb KY-ANP-II) into sites in the caudal nucleus tractus solitarii (NTS) (dark area, No. 1). No alterations in blood pressure were observed after MAb KY-ANP-II injections into surrounding areas, including hypoglossal nucleus (HN, No. 2), cuneate nucleus (CN, No. 3), spinal trigeminal nucleus (ST, No. 4), cerebellar cortex (CC, No. 5), or a position 0.6 mm ventral to caudal NTS (No. 6). AP, area postrema; DX, dorsal motor nucleus of the vagus; PT, pyramidal tract. Right panel: Longitudinal section of NTS with black circles indicating sites of microinjection of MAb KY-ANP-II. Numbers indicate distance in millimeters caudal to the interaural line.
mounted and stained with 1% thionin for verification of the microinjection site and for measurement of extent of spread of the dye.

The monoclonal antibody used in these studies was the high affinity antibody against rat α-ANP, the 28-amino acid form of ANP, produced by Mukoyama et al., and named MAb KY-ANP-II. MAb KY-ANP-II recognizes human atrial natriuretic factor (ANF) (99–128) and rat ANF equally and blocks the ability of both exogenous and endogenous ANP to elevate plasma cyclic GMP (cGMP) levels. Further, elevated plasma cGMP levels in SHR of the stroke-prone substrain (SHR-SP) and deoxycorticosterone acetate (DOCA)–salt rats were significantly reduced by intravenous administration of MAb KY-ANP-II, indicating that the antibody can block the activity of rat ANF in the intact rat. We purified IgG containing MAb KY-ANP-II from mouse ascites fluid (1 ml) using a protein A agarose column. Retained IgG with MAb KY-ANP-II was eluted from the protein A column with 3 M MgCl2 and was dialyzed against 0.9% saline overnight. We demonstrated that the purified IgG (1.1 mg/ml) with MAb KY-ANP-II, bound 50% of 125I-ANP (17,000 cpm) at 1:100,000 final dilution in a total volume of 500 μl, confirming the efficacy of the antibody in blocking ANP. In addition, we observed that intravenous injection of a 100-μg dose of purified MAb KY-ANP-II inhibited the increase in plasma cGMP induced by exogenous ANP (20 μg/kg i.v.) in intact rats, confirming the previous characterization of Itoh et al. The dose of MAb KY-ANP-II (0.55 μg) that was used in the current experiment is equivalent to the anti-ANP antibody contained in 0.55 μl of mouse ascites fluid. This is 0.5% of the peripheral intravenous dose (100 μl of ascites fluid) of this monoclonal antibody used in previous studies by Itoh et al.

Statistical Analysis

Results are expressed as mean±SEM. Two-way analysis of variance (ANOVA) (strain x time and dose x time) was performed to assess the differences in mean arterial pressure and heart rate responses to MAb KY-ANP-II between doses and between strains. One-way ANOVA was used to compare differences over time in each SHR or WKY group. Significant differences were then subjected to Newman-Keuls post hoc analysis. A value of p<0.05 was considered significant.

Results

Fifty-three SHR and 19 WKY rats were studied in all. Histological examination confirmed that in the majority of rats injected, cannulas were properly placed in the four levels of NTS and three control areas indicated in the “Methods” section. In three SHR, the cannula entered the cerebellum. In three SHR and two WKY rats, the cannula entered the brain at a position 0.6 mm inferior to the caudal NTS. In two SHR and one WKY rat, the cannula penetrated the superior cerebellar vessels; in one SHR and one WKY rat, the cannula damaged NTS tissue. These nine SHR and four WKY rats were excluded from the analysis of experimental results. Examination of 1% thionin stained sections revealed that the extent of spread of the injectate was less than 200 μm (Figure 2). Neurons near the injection tip had normal morphology in Nissl stained sections, indicating little damage at this site. SHR had significantly higher basal mean arterial pressure and lower body weight than WKY rats at the time of study (Table 1). There was no difference in basal heart rate between strains (Table 1). Microinjection of MAb KY-ANP-II into the caudal NTS resulted in significant increases in mean arterial pressure in SHR but not in WKY rats (left panel, Figure 3). In SHR, the pressor responses to MAb KY-ANP-II began almost immediately after injection, reached maximal levels at 5 minutes, and returned to baseline by 40 minutes postinjection. In contrast, microinjection of MAb KY-ANP-II into the caudal NTS did not alter heart rate significantly in either SHR or WKY rats (right panel, Figure 3).

Table 1. Basal Levels of Mean Arterial Pressure, Heart Rate, and Body Weight

<table>
<thead>
<tr>
<th>Strain</th>
<th>MAP (mm Hg)</th>
<th>HR (bpm)</th>
<th>BW (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHR (n=44)</td>
<td>165.2±1.7*</td>
<td>390.5±5.2</td>
<td>232.8±1.6*</td>
</tr>
<tr>
<td>WKY (n=15)</td>
<td>112.3±1.5</td>
<td>376.0±9.8</td>
<td>257.3±2.4</td>
</tr>
</tbody>
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Values are mean±SEM. MAP, mean arterial pressure; HR, heart rate; BW, body weight; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats.

*p<0.01 compared with WKY rats.
In SHR, microinjection of MAb KY-ANP-II into the mid-region of NTS produced pressor responses that were smaller in magnitude but not delayed in onset compared with the caudal NTS (Figure 4). Microinjection of the antibody into mid-rostral NTS caused minimal increases in mean arterial pressure in SHR (Figure 4); microinjection of MAb KY-ANP-II into rostral NTS (Figure 4) or the hypoglossal nucleus, the spinal trigeminal nucleus, or the cuneate nucleus (Figure 5) did not cause significant blood pressure responses in SHR.

Microinjection of control IgG into the caudal NTS did not alter mean arterial pressure or heart rate significantly in either SHR or WKY rats (Figure 6). Microinjection of muscimol (20 µg) into the caudal NTS significantly increased mean arterial pressure but did not affect heart rate in WKY rats (Figure 7). This is consistent with the report of Catelli and Sved\textsuperscript{15} that microinjection of the same dose of muscimol into NTS caused pressor responses in anesthetized SHR and WKY rats, verifying the responsiveness of the system.

**Discussion**

The present study demonstrated that microinjection of MAb KY-ANP-II into the caudal NTS pro-

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**Figure 3.** Line graphs show effects of microinjection of monoclonal antibody (MAb) to atrial natriuretic peptide into caudal nucleus tractus solitarii on mean arterial pressure (MAP) and heart rate (HR) in spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. *p<0.01 comparison between SHR and WKY rats over first 30 minutes (two-way analysis of variance), #p<0.05, ##p<0.01 compared with time 0.

**Figure 4.** Line graphs show effects of microinjection of monoclonal antibody (MAb) to atrial natriuretic peptide into caudal nucleus tractus solitarii (NTS), mid-NTS, mid-rostral NTS, and rostral NTS on mean arterial pressure (MAP) in spontaneously hypertensive rats (SHR). AP, area postrema. *p<0.05, ##p<0.01 compared with time 0.
FIGURE 5. Line graphs show effects of microinjection of monoclonal antibody (MAb) to atrial natriuretic peptide into the hypoglossal nucleus (N.), spinal trigeminal nucleus, and cuneate nucleus on mean arterial pressure (MAP) and heart rate (HR) in spontaneously hypertensive rats (SHR). Reduced significant increases in mean arterial pressure in SHR but not in WKY rats. Control injection of an equal volume of IgG into the caudal NTS had no effect on mean arterial pressure in SHR. Further, injection of MAb KY-ANP-II into more rostral neuronal groups in the NTS and into the hypoglossal nucleus, spinal trigeminal nucleus, or cuneate nucleus did not significantly alter mean arterial pressure. These data suggest that endogenous ANP in NTS may be involved in the centrally mediated regulation of blood pressure in SHR.

The finding that blockade of endogenous ANP in the caudal NTS has differential effects on blood pressure in SHR versus WKY rats is consistent with several lines of published evidence that brain ANP is altered in SHR compared with normotensive control rats. Studies from a number of laboratories have demonstrated that the ANP content of the hypothalamus, pons, and septum is significantly elevated in SHR compared with age-matched WKY controls. The ANP content of whole hypothalamus in young prehypertensive SHR is not significantly different from WKY rats, but hypothalamic ANP decreases with age in WKY rats and not in SHR, reaching significantly different levels by 8 weeks of age, at which time blood pressure in SHR is significantly greater than in WKY controls. Intravenous injection of ANP into the intact rat or application of ANP to brain slices in vitro causes marked increases in cGMP levels in hypothalamus and brain stem of SHR that are significantly greater than those in WKY controls. These observations suggest that ANP-sensitive particulate guanylate cyclase activity in the hypothalamus and brain stem is greater in SHR than in WKY rats. The current study was designed to test the functional significance of these alterations in endogenous brain ANP in cardiovascular regulation.

Previous studies of the centrally mediated cardiovascular effects of ANP have generally used the indirect strategy of injecting exogenous ANP into the cerebral ventricular system or into discrete brain nuclei and observing the effects of the intervention on blood pressure and heart rate. These studies have yielded inconsistent results, mainly because of differences in species of peptide, site, dose, and schedule of administration used. Administration of human ANP [human ANF-(99-126) or human ANP-(1-25)] into the lateral ventricle did not alter mean arterial pressure or heart rate in SHR or normotensive Wistar or WKY control rats, whereas injection of rat ANF significantly increased mean arterial pressure and heart rate in conscious SHR and WKY rats. The pressor and tachycardic responses to rat ANF were greater in SHR than in WKY controls. In contrast, injection of atriopeptin III [rat ANP-(5-28)] into the lateral ventricle did not alter mean

FIGURE 6. Line graphs show effects of microinjection of immunoglobulin G (IgG) into caudal nucleus tractus solitarii on mean arterial pressure (MAP) and heart rate (HR) in spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats.
Mechanisms that have been hypothesized to explain the depressor effect of blocking endogenous ANP in the AHA include direct effects on neuronal excitability and on neurotransmitter release. ANP has been shown to reduce neuronal excitability in the rat hypothalamus. When applied to individual hypothalamic neurons, ANP inhibits neuronal firing rate. Further, ANP inhibits norepinephrine release from peripheral sympathetic nerve terminals and from PC 12 cells in culture. Endogenous ANP in the AHA could participate in blood pressure control via regulation of norepinephrine release by AHA nerve terminals. Both endogenous and exogenous norepinephrine act in AHA to lower blood pressure and heart rate via an \( \alpha_2 \)-adrenergic receptor-mediated effect, presumably by inhibiting sympathetic outflow. Microinjection of norepinephrine or the \( \alpha_2 \)-adrenergic agonists clonidine and guanabenz into AHA lowers blood pressure and heart rate in the intact, conscious rat. This effect is exaggerated in the SHR compared with normotensive controls. Reductions in endogenous noradrenergic activity in the AHA would be expected to decrease inhibition of sympathetic outflow and thereby cause blood pressure to rise. Thus, we concluded that increased endogenous ANP levels in AHA were associated with inhibition of local endogenous norepinephrine release, leading to decreased sympathoinhibition and increased peripheral sympathetic nervous system activity and blood pressure in the SHR. We postulated that microinjection of the blocking antibody to ANP into AHA enhanced local norepinephrine release, thus inhibiting sympathetic outflow and lowering blood pressure.

In contrast, microinjection of ANP into NTS has been shown to produce significant increases in firing rate of NTS neurons associated with reductions in arterial pressure in anesthetized Wistar rats. Further, single units excited by microinjection of ANP into NTS are also excited by activation of arterial baroreceptors and inhibited by baroreceptor unloading. These findings suggest the possibility that ANP-induced activation of NTS neurons may mediate the depressor effect associated with arterial baroreceptor reflex activation. The NTS, which lies in

**Figure 7.** Line graphs show effects of microinjection of muscimol into caudal nucleus tractus solitarii on mean arterial pressure (MAP) and heart rate (HR) in Wistar-Kyoto (WKY) rats.

arterial pressure, whereas injection of the same dose of atriopeptin III into the fourth ventricle caused a significant decrease in mean arterial pressure but no change in heart rate in conscious SHR and WKY rats, suggesting that the site of depressor action of ANP was in the hindbrain. Microinjection of rat ANP-(5-27) into NTS, cuneate nucleus, or the spinal trigeminal complex produced significant decreases in mean arterial pressure and heart rate in anesthetized Wistar rats. No responses were found when the same dose of rat ANP was injected into the medial longitudinal fasciculus, hypoglossal nucleus, area postrema, or dorsal motor nucleus of the vagus. In contrast, microinjection of atriopeptin III into the preoptic suprachiasmatic nucleus produced significant increases in mean arterial pressure and heart rate in Sprague-Dawley rats. Taken together, these data suggest that central injection of exogenous rat ANP but not human ANP alters mean arterial pressure and heart rate in the rat and that ANP acts at hindbrain sites, especially NTS, to lower blood pressure and at anterior hypothalamic sites to elevate blood pressure.

A recent study from our laboratory has provided the first direct evidence that endogenous ANP in a region of brain known to influence cardiovascular function mediates blood pressure and heart rate control in the rat. In this study, purified MAb KY-ANP-II (0.055 and 0.55 \( \mu \)g) (the same blocking monoclonal antibody to ANP used in the current investigation) or control mouse IgG in 200 nl saline was microinjected into the anterior hypothalamic area (AHA) of conscious SHR and control WKY rats. As a further control, MAb KY-ANP-II (0.55 \( \mu \)g) was microinjected into the posterior hypothalamic area (PHA) of SHR. Anterior hypothalamic microinjection of MAb KY-ANP-II caused significant dose-related decreases in mean arterial pressure and heart rate in SHR but not in WKY rats. Control injections of an equal volume of IgG had no effect on mean arterial pressure or heart rate. Microinjection of MAb KY-ANP-II into PHA produced no significant alteration in mean arterial pressure or heart rate.
the dorsal medulla, is the primary site of termination for carotid and aortic baroreceptor afferents.36 Lesion or transection of peripheral inputs to NTS produces fulminating hypertension11,21,39-41 due to increases in sympathetic nervous system activity and vasopressin release, and activation of NTS neurons by electrical stimulation causes decreases in blood pressure.42,43 ANP and its receptors have been described previously on cell bodies and nerve terminals in the NTS,210 but our finding that blockade of endogenous ANP in the caudal NTS with MAb KY-ANP-II caused rapid onset pressor responses in SHR provided the first direct evidence that endogenous ANP in NTS is functionally significant in blood pressure control. The pressor response to microinjection of the anti-ANP antibody decreased progressively in magnitude with increasing distance rostral in the NTS. This is consistent with the observation of Ermirio et al13 that the majority of ANP-responsive sites (86%) are located between 0.5 mm rostral and 1.5 mm caudal to the obex, corresponding to the site of termination of baroreceptor and chemoreceptor afferents.

In the current study, control injections of MAb KY-ANP-II into sites immediately dorsal, ventral, and lateral to the target site in caudal NTS produced no significant effect on mean arterial pressure in the SHR (Figure 1). The very small (=200 μM; Figure 2) diffusion area of our microinjections makes it unlikely that the observed effects were due to diffusion of antibody from the injection site into surrounding areas. Further, control injections of 50 nl IgG in ACSF into the caudal NTS did not affect mean arterial pressure in SHR, ruling against the possibility that the pressor response to MAb KY-ANP-II was related to mechanical stimulation of neurons in this area. The volume injected in our experiments (50 nl) was considerably smaller than that reported to cause mechanical stimulation of the NTS (300 nl) in previous microinjection studies,44 and histological examination of thionin stained sections of microinjected brains confirmed the absence of major damage to neurons near the injection site. Our ability to deliver microinjections into the NTS of conscious, freely moving rats without creating lesions due to movement of the brain stem against a rigid injection cannula was due in major part to positioning of the indwelling guide cannula 2 mm dorsal to the NTS. The inner cannula used for the actual microinjections was very small [32-gauge stainless steel tubing] and was inserted into the brain only minutes before the acute experiment, thus minimizing the risk of mechanical trauma related to brain movement. Our cannulation technique was a modification of the procedure of Michelini and Bonagamba,45 which has previously been used successfully to assess the effects on baroreceptor reflex function of microinjecting exogenous vasopressin into NTS in conscious rats. We modified this procedure by developing a stereotaxically guided method using adjustment of the incisor bar to introduce the guide cannula, thus enhancing the accuracy and reproducibility of cannula placement and increasing the ease of anchoring the outer cannula to the skull.

Taken together, the presence of ANP binding sites and ANP-containing nerve fibers in NTS, the observation that exogenous ANP excites NTS neurons that receive a baroreceptor input and thereby elicits a depressor response, and our finding that blockade of the effects of endogenous ANP on these neurons causes a pressor response in SHR give strong evidence that ANP acts as a neurotransmitter or has another function in transmission of baroreceptor information in NTS. The pressor response to blockade of endogenous ANP in NTS occurred in SHR but not in WKY rats, suggesting that NTS neurons may be tonically activated by ANP in SHR but not in WKY rats. Increased tonic activation of NTS neurons by ANP would tend to lower blood pressure, thus representing a sort of inadequate compensation for the elevated blood pressure in SHR. Tonic activation of the baroreceptor reflex pathway in SHR under resting conditions could account for the reduced sensitivity of both cardiopulmonary and arterial baroreceptor reflexes to stimulation by volume expansion and phenylephrine infusion, respectively, in SHR compared with WKY rats.66,47 This could represent the central defect in the baroreceptor reflex pathway previously described in SHR.48 Further study, including an assessment of tonic release of ANP from nerve terminals in the caudal NTS, and determination of the effects of reducing and augmenting local ANP concentrations on baroreceptor reflex function are needed to test this hypothesis.

References


Key Words: • monoclonal antibodies • atrial natriuretic peptides • microinjections • central nervous system • brain • nucleus tractus solitarii
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